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ATP-triggered ADP release from the asymmetric chaperonin GroEL/GroES/ADP_7 is not the rate-limiting step of the GroEL/GroES reaction cycle

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ABSTRACT

The GroEL/GroES protein folding chamber is formed and dissociated by ATP binding and hydrolysis. ATP hydrolysis in the GroES-bound (*cis*) ring gates entry of ATP into the opposite unoccupied *trans* ring, which allosterically ejects *cis* ligands. While earlier studies suggested that hydrolysis of *cis* ATP is the rate-limiting step of the cycle ($t_{½} \sim 10$ s), a recent study suggested that ADP release from the *cis* ring may be rate-limiting ($t_{½} \sim 15$–20 s). Here we have measured ADP release using a coupled enzyme assay and observed a $t_{½}$ for release of $\leqslant$4–5 s, indicating that this is not the rate-limiting step of the reaction cycle.

1. Introduction

During GroEL–GroES-mediated protein folding, the two GroEL rings alternate as GroES-bound folding chambers, directed by a cycle of ATP binding and hydrolysis [1,2]. In particular, the rings are strongly anti-cooperative for ATP binding, such that only one ring at a time becomes cooperatively filled with ATP in its seven equatorial sites [3]. ATP binding to a ring rapidly frees its apical domains from each other, producing small apical domain elevation and twisting movements [4], enabling efficient polypeptide binding [5], as well as subsequent recruitment of GroES [6–8]. Association of GroES in turn triggers large apical domain elevation and clockwise twisting movements that are associated with ejection of polypeptide into what becomes, over ~1 s, a GroES-domed hydrophilic chamber where polypeptide commences to fold in isolation [9–14]. After ~10 s, this ring hydrolyzes ATP, gating entry of ATP into the opposite ring, which triggers rapid allosteric release of both GroES and polypeptide ligands from the folding-active ring [1,15]. Such ATP binding to the *trans* ring also initiates production of a folding-active state of what had been the unoccupied ring [2].

While rapid release of GroES, polypeptide, and the *cis*-formed γ-phosphate by *trans* ATP binding have been experimentally demonstrated ($t_{½} < 1$ s) [2,10,16], the rate of *cis* ADP release had not been measured until recently, but had been assumed to be similar. Thus, from measurements taken to date, it has appeared that the longest step of the reaction cycle is the folding-active, ATP/GroES-bound state of a ring (e.g. [2]), but this has recently been challenged by an experiment that reported relatively slow release of hydrolyzed ADP from the folding-active ring, measured by a gel filtration assay [17], with a half-time of release of ADP of 15–20 s. Prolonged retention of ADP would sterically prevent ATP from rebinding to the original *cis* ring when the machine returns from a subsequent cycle on the opposite ring. Even more immediately, ADP might allosterically inhibit the step of ATP turnover in the next cycle occurring in the opposite ring (e.g. [18]). Given this measured dwell time of ADP, its release was proposed to be the rate-limiting step of the reaction cycle [17]. Yet a rate-limiting step with a $t_{½}$ of 15–20 s seems inconsistent with previously measured rates of steady-state ATP hydrolysis of actively cycling GroEL–GroES reactions, which correspond, as mentioned, to a $t_{½}$ of ~10 s (e.g. [2,19]). Because of concerns about the reported measurement, we have measured this step by a different method, employing a coupled enzyme assay that provides a fluorescent readout of the rate

Abbreviations: DTT, dithiothreitol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); LDH, lactate dehydrogenase; PEP, phosphoenol pyruvate; PK, pyruvate kinase

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of ADP departure following ATP binding to the *trans* ring of an asymmetric GroEL/GroES/ADP_7 complex. We report that, by such measurement, ADP release is complete within 10 s. Thus ADP release does not appear to be rate-limiting and rather, it would appear, as in earlier observations, that it is *cis* ATP hydrolysis that is the rate-limiting step of the reaction cycle.

2. Materials and methods

2.1. Proteins

GroEL D398A was expressed in *Escherichia coli* and purified as previously described [2]. Asymmetric GroEL D398A/GroES/ADP_7 complexes were generated by mixing 10 μM GroEL D398A with 12 μM GroES and 70 μM ATP in 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), pH 7.5, 5 mM KOAc, 10 mM $Mg(OAc)_2$, 1 mM dithiothreitol (DTT), and incubating for 2 h at 25 °C. Efficiency of formation of these complexes and stability during incubation was assessed by gel filtration studies using a fluorescent-labeled GroES (see Supplementary figure).

2.2. ADP release assay

ADP release from chaperonin complexes was measured by coupling with pyruvate kinase and lactate dehydrogenase, following the decrease in NADH fluorescence [20]. In all experiments, NADH was excited at 340 nm, and emission was measured using a color separation filter (370–525 nm). In a typical ADP release experiment, equal volumes of 10 μM D398A/GroES/ADP_7 complex and assay buffer (50 mM HEPES, pH 7.5, 5 mM KOAc, 10 mM $Mg(OAc)_2$, 1 mM DTT, 0.5 mM phosphoenol pyruvate (PEP), 0.150 mM NADH, 120 μg/ml pyruvate kinase (PK), 80 μg/ml lactate dehydrogenase (LDH)) containing 0.3 mM ATP were mixed in a stopped-flow device and fluorescence recorded. The data from at least 10 individual mixes were summed for each kinetic trace.

3. Results and discussion

To measure the rate of ADP release from an asymmetric GroEL/GroES/ADP_7 complex, a mutant version of GroEL, D398A, was employed [1]. The substitution in this mutant, located in the intermediate domain, alters the residue that serves as a base to activate water for ATP hydrolysis. The mutant hydrolyzes ATP at a rate ~2% that of wild-type GroEL, and thus effectively allows study of a single turnover of the reaction. Accordingly, D398A was incubated with GroES and a concentration of ATP sufficient to fill one ring, allowing formation of an asymmetric GroEL398A/GroES/ATP_7 complex (Fig. 1). This complex was incubated for 2 h at 25 °C to allow ATP in the *cis* ring to hydrolyze, producing an ADP-containing asymmetric complex whose ADP release could now be studied by stopped-flow mixing with ATP, which would bind to the open *trans* ring, discharging the *cis* ligands [1]. The rate of departure of ADP from the *cis* ring was monitored here by a coupled enzyme assay using pyruvate kinase and lactate dehydrogenase (Fig. 1). The pyruvate kinase step transfers a phosphate from PEP to the released ADP to form pyruvate and ATP, and the lactate dehydrogenase (LDH) step reduces the pyruvate to lactate via the oxidation of NADH, whose loss of fluorescence could be directly monitored.

In a control test, when ATP was added to GroEL D398A alone (Fig. 2), we observed no change in the NADH fluorescence over 40 s, consistent with very slow turnover of ATP by the D398A mutant complex and reflecting the ability of the assay to distinguish ADP from ATP. In a second set of control tests, varying known concentrations of ADP similar to those expected to be produced were directly added to the assay mixture in the absence of chaperonin by stopped-flow mixing (Fig. 3). This produced in each case a decline of NADH fluorescence after a very brief lag, to an extent proportional to the concentration of ADP (Fig. 3). In this range of

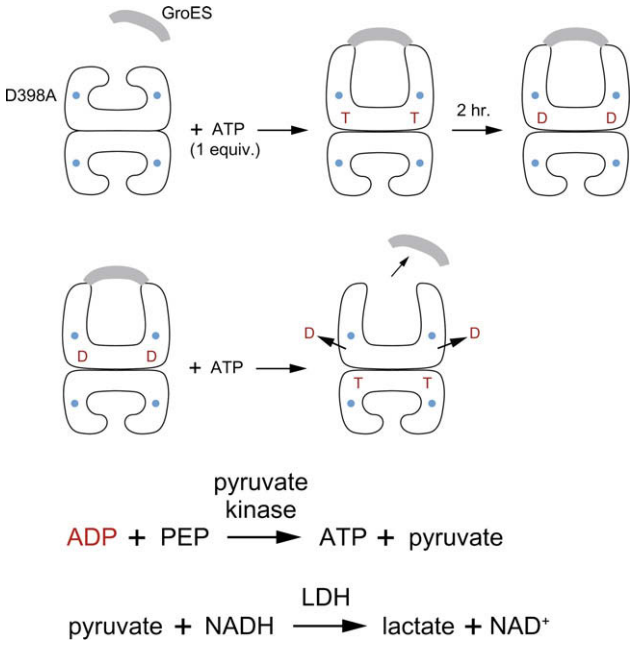

Fig. 1. Scheme for monitoring ADP release from asymmetric GroEL/GroES/ADP_7 complex. D398A ATP hydrolysis-defective GroEL (10 μM) was employed to confine the reaction to a single turnover. An asymmetric complex with GroES (10 μM) was formed (top row) by adding 70 μM ATP amounting to 1 ring-full, producing an asymmetric D398A/GroES/ATP_7 complex. This was allowed to undergo ATP hydrolysis during a 2 h incubation to produce a D398A/GroES/ADP_7 complex. The asymmetric ADP complex was then incubated with ATP (150 μM final concentration) in a coupled enzyme assay mixture by stopped-flow mixing (second row). Binding of ATP in the *trans* ring leads to ejection of the ligands from the *cis* ring (second row right panel). Released ADP was detected by the coupled enzyme mixture (third and fourth rows), with loss of NADH fluorescence monitored over time. Blue dots signify D398A substitution in intermediate domain of the GroEL subunits: T, ATP; D, ADP; PEP, phosphoenolpyruvate; and LDH, lactate dehydrogenase.

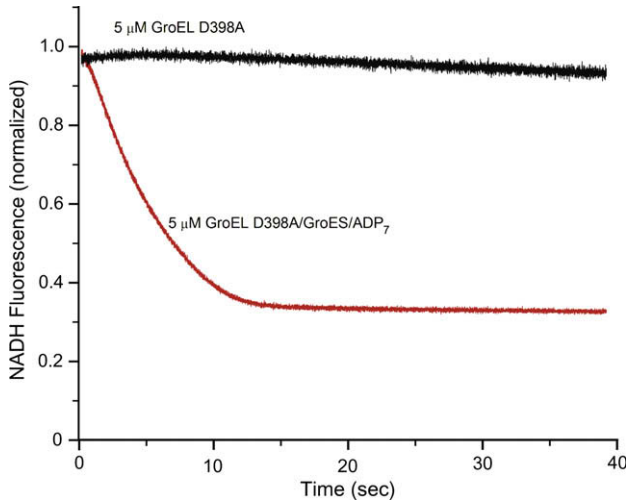

Fig. 2. ADP release from GroEL D398A alone (black trace) and from a D398A/GroES/ADP_7 complex formed as in Fig. 1 (red trace) monitored by loss of NADH fluorescence. The respective chaperonins, 10 μM, were mixed 1:1 in the stopped-flow device with the coupled assay mixture containing 300 μM ATP, and fluorescence of NADH was directly monitored.

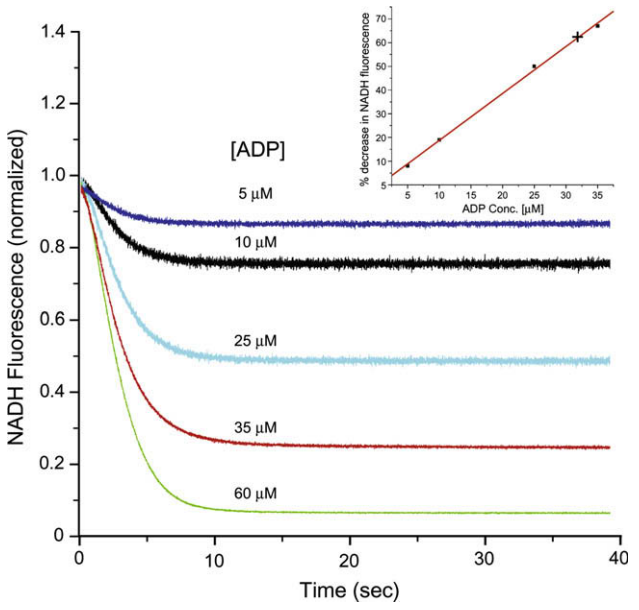

Fig. 3. Measurement of rate and extent of NADH consumption upon stopped-flow mixing of various concentrations of ADP with the coupled assay mixture. For all concentrations used, there is a drop in fluorescence beyond a slight lag that could be fit with a single exponential, with a rate constant of ~0.30 s^{-1}. The extent of consumption increases linearly with the concentration of ADP (see inset, where "+" indicates the extent of the reaction in Fig. 2). Note that the rate of fluorescence change in the assay itself is faster than that produced upon incubation of the asymmetric complex with ATP (Fig. 2), allowing use of the coupled assay to determine a rate of ADP release from the asymmetric complex (see text).

concentrations, the rates of NADH oxidation could be fit to single exponential equations (disregarding the initial lag) with similar apparent rate constants of about 0.30 s^{-1}. These data indicate that any observed rates of ADP release slower than 0.30 s^{-1} would represent actual rates of release (rather than rates of the coupling reactions). Next, 300 μM ATP was added to 10 μM GroEL398A/GroES/ADP_7 complex by 1:1 stopped-flow mixing (Fig. 1). This produced a drop of NADH fluorescence (after a slight lag) with an apparent rate constant of 0.15 s^{-1} (Fig. 2). Because this rate is slower than the rate of the coupling reaction, it provides at least a minimum estimate of the rate of ADP release in an ongoing cycling reaction. The amplitude of the change in Fig. 2 corresponds to ~32 μM ADP (cf. Fig. 3 inset), close to the amount expected for turnover of a single ring (35 μM). Thus the present measurements support a half-time of ADP release of less than 4–5 s, indicating, e.g. in comparison to the ~10 s half-time for ATP hydrolysis, that this is not the rate-limiting step of the reaction cycle.

Despite the use here of a mutant GroEL complex, D398A, to allow a single turnover report on the rate of ADP release from a discharging asymmetric GroEL/GroES complex, we expect the rate measurement would extrapolate to wild-type GroEL. This is based on two observations. First, the two complexes exhibit similar affinities for GroES in the presence of ADP. Second, the two complexes exhibit similar kinetics of ATP-mediated discharge of the GroES ligand, likely reflecting similar structural changes across the ring–ring interface and through the three domains of the discharging ring (see Supplementary discussion). As a further comment, we note that in additional measurements of ADP release with the D398A system, neither the presence of *trans*-bound substrate protein (Rubisco or MDH) nor *cis* folding substrate (MDH) affected the rate of ADP release. Thus, in contrast with the step that precedes ATP-triggered dissociation of the asymmetric ADP complex, shown to be accelerated by substrate protein binding to the *trans* ring [2], the step of ATP-triggered dissociation of ADP does not appear to be sensitive to the presence of substrate protein.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.01.021.

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